Two-Dimensional Contrast Echocardiography. I. In Vitro Development and Quantitative Analysis of Echo Contrast Agents

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To facilitate the passage of echo contrast agents through the microcirculation and the echocardiographic study of myocardial perfusion, ultrasonic energy (sonication) was employed to produce contrast agents consisting of relatively uniform, stable and small (< 10 μ diameter) gaseous microbubbles suspended in liquid solutions. The size and persistence of the microbubbles was verified by light microscopy and an in vitro system were employed for comparative assessment of peak echo amplitude and echo persistence characteristics of various contrast agents. The study indicated that although a variety of hand-agitated and sonicated contrast agents provided satisfactory echo intensities, sonication was clearly superior to the hand-agitation method, because sonication produced smaller, more uniform and more stable microbubbles that may be suitable for myocardial contrast echocardiography.

It is concluded that of the contrast agents examined, sonicated solutions of sorbitol (70%) and dextrose (70%) appeared to have particular potential because of the small sizes of the microbubbles (6 ± 2 and 8 ± 3 μ, respectively) and their prolonged in vitro persistence. The use of sonication to produce standardized, small and stable microbubbles should facilitate physiologic passage of the contrast agent through the capillary beds and allow two-dimensional imaging of the left heart myocardium during right-sided, aortic root, coronary sinus or intracoronary contrast injections.

Since the initial observations of Gramiak and Shah (1) in 1968, contrast echocardiography has been used to delineate intracardiac structures, assess valvular competence, demonstrate intracardiac shunts and identify pericardial effusion (2) Most recently, Armstrong et al (3), Meltzer et al (4) and Tei and associates (5) from this laboratory demonstrated the use of contrast echocardiography to visualize left ventricular myocardium and areas of myocardial underperfusion during experimental coronary artery occlusion However, application of contrast echocardiography to quantitative assessment of myocardial perfusion in normal and ischemic regions of the myocardium requires a more comprehensive understanding of the characteristics of contrast agents and their effects within the myocardial microcirculation Development of standardized echo contrast agents, such as those containing uniformly small and stable gaseous microbubbles, appears to be an important prerequisite for further progress in the new field of myocardial contrast echo imaging

The present report describes our efforts to examine and develop a variety of contrast agent solutions containing microbubbles that can be produced in a practical and controlled manner and would be sufficiently small and stable to readily traverse the capillary beds Supplemental ongoing investigations examine the newly developed agents through microscopic dynamic studies of the microcirculation, and preliminary data on transpulmonary passage of such agents are described in an accompanying report (6)

Methods

Materials. We examined a variety of echo contrast agents, including those previously reported (7,8) The solutions analyzed included

1 Degassed tap water

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2 Dextrose 5% in water
3 0 9% NaCl
4 Renografin-76
5 Indocyanine green
6 Hydrogen peroxide
7 Carbonated saline solution
8 70% sorbitol
9 Dextrose 70% in water
10 Dextrose 50% in water
11 Mixture—1 1 ratio 25% mannitol and 0 9% saline solution
12 Mixture—1 1 ratio of Renografin-76 and 0 9% saline solution
13 Mixture—1 1 ratio of 70% sorbitol and dextrose 5% in water

Methods of preparation. *Hand agitation* With two syringes connected by a three-way stopcock, mixtures of certain solutions were forcibly flushed back and forth approximately 15 to 20 times, until sufficient agitation was produced A small amount of air was introduced into a syringe before the flushing to serve as a source of air for microbubble development

Sonication The Heat Systems sonicator model W-375 with a lead zirconate-titanate electrostrictive (piezoelectric) crystal served as a controlled source of ultrasonic energy Introduction of the tip of the sonicator horn into a solution resulted in the production of surface agitation and cavitation, which created microcavities within the liquid as a result of the powerful ultrasonic compressions and rarefactions After the cavitation bubbles collapsed, immediately on cessation of the ultrasonic energy, a second generation of bubbles was created from the released cavitation gas bubbles (9) Solutions containing dissolved gas (that is, carbonated saline and hydrogen peroxide) were not sonicated, because large amounts of gas bubbles were already visibly present

Microscopic analysis. *Bubble size* To examine the microbubbles after preparation, one drop of the previously prepared solution was sampled from the middle of the syringe and then rapidly placed onto a microscope slide with a cover slip Before placing a drop of the solution on a slide, 2 ml of the newly prepared solution was pushed through the stopcock to flush out any trapped air bubbles Then by placing a single drop of a contrast agent (with a 1 ounce cover slip) under view of a binocular Olympus CHA microscope, multiple fields were viewed at a 400 magnification for 1 minute An eyepiece net reticle graduated in microns was used to visually determine bubble size to the nearest 2 μ by direct visual or photographic inspection For each observation period, a fresh solution of the agent being studied was prepared. The mode, mean and standard deviation of the bubble size were calculated

Persistence The persistence of a contrast agent was examined by maintaining and viewing one drop of the contrast agent under the microscope for 5 minutes The number of microbubbles was counted at the beginning and end of the 5 minute period, and multiple field observations were recorded for each agent Persistence, expressed as a percent, was determined by dividing the number of bubbles present at the end of 5 minutes by the number initially present.

In vitro system. An in vitro tube system was constructed to quantify peak intensity and persistence of ultrasound signals of various contrast agents The system employed a reservoir filled with degassed tap water, which drained by gravity through standard intravenous tubing connected to Tygon tubing of 3/8 inch (0.95 cm) external diameter The gravity flow was maintained at 2 cc/s Proximal to the intravenous tubing, a "Y" connector served as the site of the injection of various contrast agents into the tube system A 10 cc plastic syringe with a 1 inch (2 54 cm), 20-gauge needle was utilized for each injection consisting of 8 cc of a particular contrast solution The force of injection was standardized by using a carbon dioxide-powered thermodilution injector (USCI/OMB)

Ten centimeters downstream from the injection site, the Tygon tubing was submerged in a styrofoam container filled with degassed tap water and held in a rigid position by means of a specially designed Lucite holder The ultrasonic transducer (3 MHz, ATL 300) was held in a copper cradle and focused at a distance of 7 7 cm above the Tygon tubing in the styrofoam container The position of the transducer and the Tygon tubing section to be imaged were standardized by fixing the distance vertically and establishing uniformity of the A-mode and M-mode image of the anterior and posterior walls of the tubing

For persistence studies, the Tygon tubing was cross-clamped just distal to the area of ultrasonic imaging The flow was stopped in the tubing system and the particular contrast agent was injected into the Y connector site After every injection, the entire system was flushed with degassed tap water and the A-mode and M-mode images of the anterior and posterior walls of the Tygon tubing assumed their control amplitudes The amplitude and gain settings on the echo receiver unit were standardized throughout the experimental process

The intensity of the echographic image of injected contrast agents in the flowing tube system was expressed (as a percent) as peak amplitude on the A-mode luminal image divided by the peak amplitude of the anterior wall signal of the tube (Fig 1) Frame by frame visual analysis of peak amplitudes from A-mode images was measured from a hard-copy printout using a calipers (to the nearest 1/50th of an inch)

Persistence was evaluated by the following criteria 1) presence of luminal reflected echographic images, or 2) obfuscation of the posterior wall of the Tygon tubing Persistence was checked on five separate occasions during the time that the lumen of the tube contained reflected echographic images or the posterior tubular wall could not be imaged A baseline echographic image consisting of a clear

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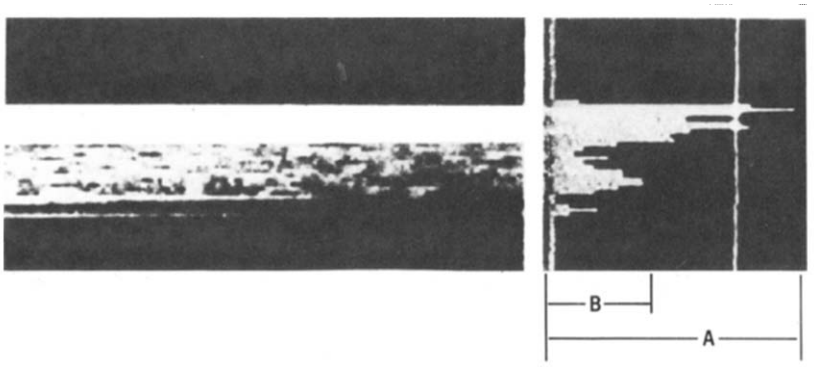

Figure 1. In vitro tube system peak amplitude analysis from an A-mode frame of a videotape recording **Left,** M-mode recording of the tube system **Right,** A-mode recording

$$\text{A-mode Peak Intensity} = \frac{\text{B (peak amplitude of the contrast agent in the lumen)}}{\text{A (constant amplitude of the anterior wall)}} \times 100$$

tubular lumen, and a posterior wall image that was established before contrast injection served as the control

Statistics. All data are expressed as the arithmetic mean ± standard deviation The microbubble diameter measurements for each group were entered into a DEC-VAX 750 computer and analyzed using an analysis of variance (ANOVA) model Because the variances of the six groups were not equivalent, the Brown-Forsythe ANOVA was used (10) The probability (p) value for the ANOVA was less than 0 001 The significance levels for differences between individual solutions were then computed using the Scheffé multiple comparisons test (11)

Results

Microscopic analysis. Table 1 lists the microscopic analysis data It shows that the sonicated sorbitol solution (70%) and the sonicated dextrose solution (70%) had the smallest mean bubble sizes and the smallest standard deviations. Also, the Renografin-76–saline mixture had a significantly smaller mean bubble size and standard deviation when sonicated than it had when it was hand-agitated The mean microbubble diameter of each solution tested was significantly different from that of each other solution ($p < 0\ 001$), except for the sonicated solutions of dextrose solution (50%) (11 ± 5 μ) and sorbitol solution (70%) diluted with dextrose 5% in water (11 ± 5 μ), dextrose solution (50%) (11 ± 5 μ) and Renografin-76 (10 ± 4 μ), and sorbitol solution (70%) diluted with dextrose 5% in water (11 ± 5 μ) and Renografin-76 (10 ± 4 μ) Because the variability of the microbubble size is also of interest, we tested the variance of bubble diameter in the sorbitol solution (70%) and dextrose solution (70%) to determine if there was a difference Using both the F test and Bartlett's test (12), the sorbitol solution (70%) was found to have a smaller variance than that of the dextrose solution (70%) ($p < 0\ 001$) From these data, we concluded that sonicated sorbitol (70%) contained smaller and more uniform microbubbles than did the dextrose solution (70%)

Figure 2 demonstrates the frequency of distribution of two of the prepared agents Note that the sonicated sorbitol solution (70%) had a smaller range of bubble sizes than did the hand-agitated Renografin-76 and saline mixture

In Figure 3, the effects of dilution on bubble size and

Table 1. Microscopic Analysis

Agent	No	Mean (μ)	Mode (μ)	Persistence (%)
Sorbitol 70% (sonicated)	354	6 ± 2	4	80%
Sorbitol 70% diluted 1 1 with dextrose 5% in water (sonicated)	346	11 ± 5	12	69%
Sorbitol 70% (hand-agitated)	211	23 ± 24	12	92%
Dextrose 70% (sonicated)	397	8 ± 3	6	57%
Dextrose 50% (sonicated)	285	11 ± 5	8	63%
Renografin-76 (sonicated)	386	10 ± 4	9	58%
Renografin-76 diluted 1 1 with saline solution (sonicated)	326	13 ± 5	12	42%
Renografin-76 diluted 1 1 with saline solution (hand agitated)	295	16 ± 13	16	50%

No = total number of bubbles sampled

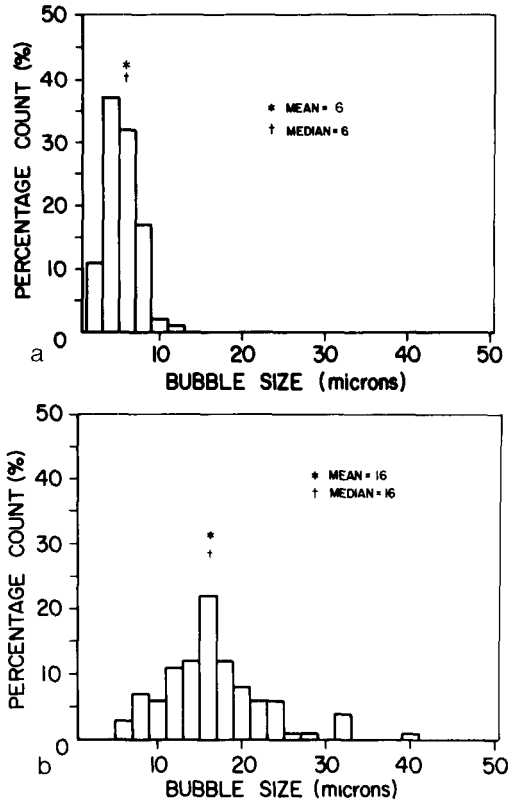

Figure 2. Histograms depicting the frequency of occurrence of microbubble sizes using two solutions and two methods of preparation **a,** Sorbitol 70% sonicated solution **b,** Renografin/saline hand-agitated solution

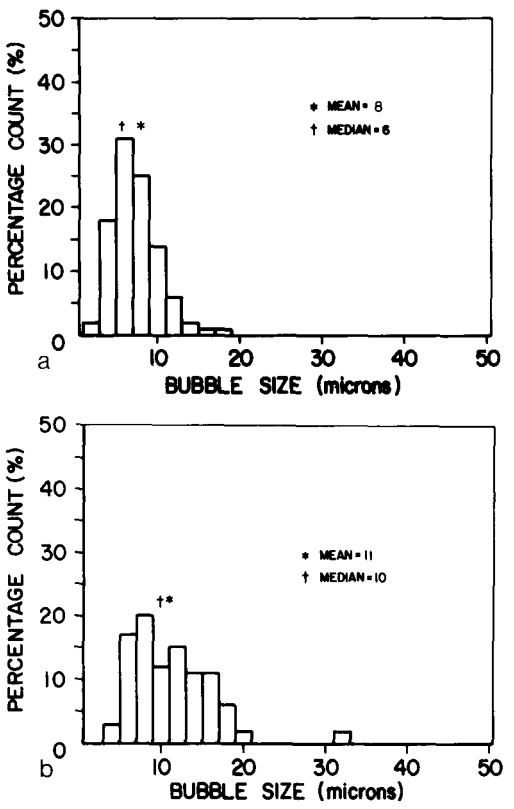

Figure 3. Frequency distribution of microbubble sizes with a dilution effect in sonicated solutions **a,** Dextrose 70% sonicated solution **b,** Dextrose 50% sonicated solution

distribution are demonstrated The sonicated dextrose solution (70%) exhibited an increase in smaller bubbles and a narrower range of bubble sizes as compared with the sonicated dextrose solution (50%) The mean size for the dextrose 70% was 8 ± 3 μ as compared with the 11 ± 5 μ mean bubble size of the dextrose 50% solution

Figure 4 demonstrates the effect of two methods of preparation of contrast agents Sonication of the Renografin-76-saline mixture produced a smaller size range of microbubbles (mean 13 ± 5 μ) as compared with a larger mean bubble size and standard deviation (16 ± 13 μ) in the same solution prepared by hand agitation

The microscopic persistence studies (Table 1) showed that the microbubbles contained in the sorbitol (70%) solution persisted over the longest time period, whereas the microbubbles contained in the diluted solutions of Renografin-76 persisted over the shortest time period

In vitro system analysis (Table 2). Mechanical agitation (either sonication or hand agitation) of solutions produced greater peak luminal echo amplitudes than those found in nonagitated solutions For example, nonagitated Renografin-76 had a peak amplitude of 63 0 ± 5 0%, but with ultrasonic agitation (sonication) it had a peak of 89 0 ± 6 7% Similarly, the peak amplitude for nonagitated sorbitol 70% in water was 62 4 ± 21 7%, and greater than 120% with agitation (sonication) Note that the sonicated Renografin-76 solution produced less peak amplitude but longer persistence than the sonicated diluted Renografin-76-saline solution

The steady state persistence studies also showed that only with contrast agent agitation did the in vitro tube luminal echo amplitude persist for any significant length of time For example, Renografin-76, when diluted to a 1 1 ratio with saline solution and then either hand-agitated or sonicated, persisted in the lumen of the tube system By contrast, without mechanical agitation, the Renografin-76-saline mixture failed to demonstrate any persistence time

The intraobserver variability for repeated examinations of the microscopic analysis of the mean bubble size was less than 2 μ (which was the smallest measurement unit), and the variability of the microscopic persistence data was less than 10% for all agents examined The day to day variability of the microscopic analysis is presented in Table 3 It was observed that the sonicated sorbitol 70% and Renografin-76 had less than a 2 μ diameter variation as assessed by day to day examination of multiple samples by one observer The variability of the peak amplitude of a contrast agent in the in vitro tube system was based on 5 to 10 individual injections and expressed as standard deviations with ranges from 3 2 to 21 7% (Table 2)

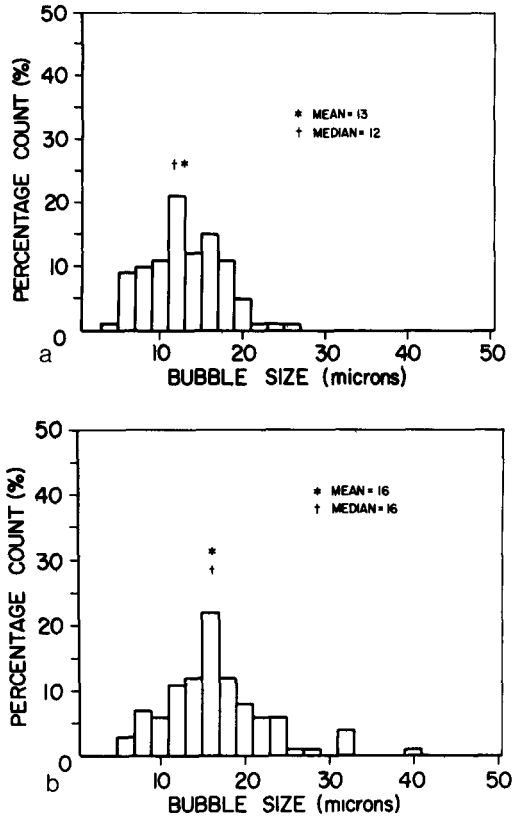

Figure 4. Effects of sonication and hand agitation on microbubble size and distribution in a solution of Renografin-76 and saline **a,** Sonicated solution **b,** Hand-agitated solution

Discussion

Sonication appears to be a readily applicable method of agitation that produces small, relatively uniform and stable microbubbles This sonication process has been used for producing finely dispersed homogeneous tissue preparations and emulsions (13) Sonication utilizes ultrasonic cavitation to create these dispersions As a by-product of this cavitation process, a dispersion of small microbubbles is formed in a solution These by-product microbubbles appear to be the source of the echo contrast effect in these solutions The sonication process occurs in a stepwise manner This process will be discussed briefly because of its new proposed application in the field of contrast echocardiography

Phases of ultrasonic sonication. This method is primarily based on a cavitation process that appears to include a *preinitiation phase* during which nuclei within the liquid (for example, a small amount of trapped air or particulate matter, or both) are brought into the intense core of the sound field transmitted from the horn tip of the sonication unit Powerful compressions and rarefactions cause these cavities in the liquid to be compressed and expanded in a cyclic manner On reaching its resonant size (presuming it is not carried away from the core position by fluid currents or mixing) the stage is set for the *catastrophic phase* This phase is characterized by violent oscillations of the resonant cavity in the stream of vibrational energy Cyclic collapse and expansion of these cavities radiate periodic shock waves in the solution, opening up microcavities that are extremely small but so plentiful that a cloudlike appearance can be

Table 2. In Vitro Tube System Analysis

	Peak Amplitude (%)	Persistence (seconds)
Ultrasonic agitation (sonication)		
70% sorbitol	>120	>180
70% sorbitol (diluted to 1 1 with dextrose 5% in water)	>120	>180
70% dextrose	110 ± 5 3	>180
50% dextrose	112 ± 2 0	>180
Renografin-76	89 ± 6 7	20 8 ± 6 2
Renografin-76 (diluted to 1 1 with saline solution)	108 ± 4 7	11 5 ± 2 8
Hand agitation		
70% sorbitol	103 ± 2 0	>180
Renografin-76 (diluted to 1 1 with saline solution)	>120	>180
Nonagitated		
Hydrogen peroxide	79 ± 9 3	<1
Carbonated saline solution	70 ± 7 0	<1
Renografin-76	63 ± 5 0	<1
Renografin-76 (diluted with saline solution)	49 ± 10 5	<1
Mannitol 25% (diluted with dextrose 5% in water)	55 ± 13 0	<1
Saline solution	43 ± 5 8	<1
70% sorbitol	62 ± 21 7	<1
Indocyanine green	81 ± 10 5	<1
Dextrose 5% in water	40 ± 8 0	<1
Dextrose 70%	82 ± 4 3	<1
Dextrose 50%	82 ± 3 2	<1

Table 3. Day to Day Variability of the Microscopic Analysis of Sonicated Microbubbles

Sample	n = (bubbles)	Mean ± Standard Deviation	Range (μ)
Sorbitol 70% (sonicated)			
Day 1			
1	48	6 ± 2	2 to 10
2	52	5 ± 2	2 to 8
3	43	6 ± 2	2 to 8
4	49	5 ± 2	2 to 10
Day 2			
5	52	6 ± 2	2 to 12
6	60	5 ± 2	2 to 8
7	50	5 ± 2	2 to 12
Total	354	6 ± 2	2 to 12
Renografin-76 (sonicated)			
Day 1			
1	45	9 ± 4	4 to 25
2	38	8 ± 3	4 to 12
3	55	10 ± 5	4 to 24
4	35	10 ± 4	4 to 20
Day 2			
5	37	10 ± 4	4 to 20
6	35	12 ± 8	4 to 32
7	39	8 ± 3	4 to 14
8	39	9 ± 3	4 to 16
9	31	8 ± 3	4 to 20
10	32	11 ± 5	4 to 20
Total	386	10 ± 4	4 to 25

noted in the solution. The next phase, *the bubble phase*, occurs simultaneously with the catastrophic phase, that is, as a by-product of the catastrophic phase, noncollapsing bubbles are created outside of the core of the energy beam. In solutions that are degassed or subject to reduced atmospheric pressures, no secondary microbubbles will be generated. Finally, a *postcavitation phase* occurs in those solutions that have sufficient nuclei present to support the preceding noncollapsing by-product microbubbles that remain in the solution after the ultrasonic energy is turned off.

Evaluation of various contrast solutions. Our application of ultrasonic energy (sonication) to a liquid solution showed that the microbubbles created in the viscous solutions such as sorbitol (70%) and dextrose (70%) were less than 10 μ in diameter (6 ± 2 and 8 ± 3 μ, respectively). The more diluted, less viscous solutions contained larger microbubbles ranging in size from 10 to 23 μ when sonicated. In the solutions prepared by both sonication and hand agitation techniques, microbubbles were significantly smaller and more uniform when produced by sonication. We are now examining the physicochemical properties of the contrast agents to identify optimal solutions that will support or stabilize small microbubbles.

The in vitro tube studies corroborated that peak echo amplitudes and persistence of the various contrast solutions were enhanced if they were agitated, either by hand or by sonication, when compared with the respective nonagitated solution (14). It is likely that the introduction of microbubbles of air into the solutions produced the enhanced ultrasonic effects. These findings are also consistent with earlier reported observations (7,8).

Limitations and advantages. Application of in vitro studies to in vivo situations may be limited to the extent that temperature, pressure, blood flow and tissue characteristics may alter microbubble size and stability. In addition, bubble size and stability may be altered by changes in temperature of the liquid during the sonication procedure, the temperature of the microscopic stage, the length of the observation period and the method of standardizing the technique for sonicating the agents.

Despite these limitations, our preliminary in vivo direct visualization investigations using the basic cat microcirculation preparation (15) with the new sonicated echo contrast agents provided promising evidence supporting the anticipated importance of the smaller size of the microbubbles. The small microbubbles (mean size 8 ± 3 μ) contained in a dextrose solution (70%) readily passed through the cat capillary microvessels, whereas the larger microbubbles contained in a hand-agitated, Renografin-76-saline echo con-

trast mixture (mean size 16 ± 13 μ) clearly caused occlusion of the microvessels and evident stoppage of individual microvascular flow Furthermore, our preliminary experiments with direct intracoronary injections of various echo contrast agents in dogs have demonstrated myocardial contrast washout rates that appear to be physiologic and significantly shorter in duration for solutions containing the sonicated, small microbubbles than the prolonged disappearance rates of 23 ± 6 seconds noted with solutions containing the larger microbubbles obtained using the hand-agitation method (16)

In the past, the left heart structures could not be consistently visualized using right heart injection sites because of microbubble instability and relatively larger sizes (17) It would appear from our initial in vitro studies and preliminary in vivo work that sonication provides a simple controlled method of agitation that, in certain solutions, will produce small, relatively uniform and stable microbubbles These sonicated agents may prove useful in contrast echocardiographic imaging of left heart structures via right heart injection sites

Future applications. Recent interest in contrast echocardiography has been heightened by potential applications to the study of myocardial defects, and ultimately to the quantification of myocardial perfusion (3–5) Preliminary indications are that adequate videodensitometric methods (18) and indexes derived by computer algorithms (16) can now be utilized for experimental assessment of myocardial contrast echocardiographic measurement of regional myocardial perfusion or underperfusion, in a manner analogous to methods using nuclear imaging

We introduced sonication as a new method generating a narrow range of small (< 10 μ diameter) microbubbles We now anticipate that it will be possible to produce, in a standardized manner, small and persistent contrast agent microbubbles capable of passing through the microvasculature, permitting echocardiographic visualization of the left ventricular myocardium, preferably from a right heart or coronary sinus injection site Thus, assuming that future research will demonstrate the safety and effectiveness of the new small microbubble-containing contrast agents, it is anticipated that an important new echocardiographic method will become available for dynamic simultaneous measurement of regional myocardial contractile function *as well as* perfusion

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